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scDam&T-seq [version half volumes]

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We use this protocol and it's working

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Abstract

An adapted version of the scDam&T-seq multiomic single-cell method (Rooijers et al., 2019, Markodimitraki et al. 2020) with reduced reaction volumes.

This improved protocol was used by Kefalopoulou et al., 2025 to generate joint single-cell measurements for Dam-LMNB1 and transcriptome during *in vitro*-induced X inactivation (Figure 5).

Attachments



DamT_Dispensions_Rob.

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21KB



Materials

For the complete list of materials to perform scDam&T-seq, check [Markodimitraki et al., 2020](#).

Materials for the adapted steps described in this version are listed here:

Oligonucleotides

- Adapters, top and bottom oligonucleotides, set of 384 (Markodimitraki et al., 2020)



DamID2_adapters_384_top_bottom... 36.1KB

- CELseq2 primers, set of 384 (Markodimitraki et al., 2020)



CELseq2_primers_384_annotated.x... 23KB

- Random hexRT primer

GCCTTGGCACCCGAGAATTCCANNNNNN

- Illumina RNA PCR primer 1 (RP1)

- Illumina RNA PCR index primers (RPI series)



RPI_primers.xlsx 10.1KB

Chemicals and Buffers

- Igepal CA-630 (Sigma, I8896-50ML)
- DTT 1M (Invitrogen, Y00147)
- dNTP set 100mM (Invitrogen, 10297018)
- 10X rCutsmart buffer (NEB, B6004)
- First-strand buffer (Thermofisher)
- Second-strand buffer (Thermofisher)
- T4 Ligase buffer (Roche)
- Mineral Oil (Sigma-Aldrich, M8410)
- Bead-binding buffer (1M NaCl, 20% PEG8000, 20mM Tris-HCl pH = 8, 1mM EDTA)
- Fragmentation buffer (500mM potassium acetate, 150mM magnesium acetate, 200mM Tris-acetate)
- CleanNGS DNA and RNA purification beads (GC Biotech, CNGS-0050)

Enzymes

- Proteinase K solution 20 mg/ml (Ambion, AM2548)
- DpnI (NEB, R0176L)
- T4 DNA Ligase (Roche, DNALIG-RO)
- Superscript II Reverse Transcriptase (Thermofisher, 18064071)
- RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen, 10777019)



- Ribonuclease H (ThermoFisher, 18021071)
- E. coli DNA Ligase (NEB, M0205L)
- DNA polymerase I (ThermoFisher 18010025)
- Phusion High Fidelity 2X PCR mastermix (NEB)

Commercial Assays

- MEGAscript T7 transcription kit (Invitrogen, AMB13345)
- Agilent High Sensitivity DNA Assay (Agilent, 5067-4626)
- Agilent RNA 6000 Pico Assay (Agilent, 5067-1513)
- Qubit sdDNA High Sensitivity Assay (Invitrogen, Q32854)

Other

- Hard-Shell 384-well PCR plates (Bio-Rad, HSP3801 or PerkinElmer, 6008910)
- 384 PCR machines
- VBLOK200 Reservoir (Click-Bio, CBVBLOK200S)
- Low-retention pipette tips (Greiner Bio-One)
- Polypropylene round bottom tubes 5 ml (Corning, 352002)
- PCR plate seals (Greiner, 676090)
- Qubit 4 fluorometer (Invitrogen)
- Agilent 2100 Bioanalyzer platform

FACS

- BD FACS Influx Cell Sorter System
- BD FACS Jazz Cell Sorter System

Robotic liquid handling

- Nanodrop II liquid handling platform (Innovadyne)
- Mosquito LV liquid handling platform (STP Labtech)
- Freedom EVO liquid handling platform (Tecan)

Sequencing

- Illumina NextSeq500 sequencing platform
- Illumina NextSeq2000 sequencing platform



Before start

Check the protocol by [Markodimitraki et al., 2020](#) for a detailed description of all steps in scDam&T-seq. That protocol contains details in regard to DamID construct design, sample preparation for FACS, and primer/adaptor design.

Here we describe only the steps adapted from the original scDam&T-seq version, which are the dispensation volumes performed by liquid handlers, to facilitate a more cost-friendly protocol. We also attach for convenience the procedure for pooling, amplification and library preparation, that remain the same as in the original version.

Preparation

1 Prepare 384-well plates containing CELseq2 primers



1. Using the Freedom EVO liquid handling platform (Tecan) or equivalent, dispense 384-well plates with 5 μ L per well mineral oil. Can be stored at room temperature, protected from light, until use.
2. Using the Mosquito LV (STP Labtech) or equivalent, dispense **50 nL per well** of 1500nM CELseq2 primers from a CELseq2 motherplate. Can be stored at -20°C until use.

Single-cell sorting (FACS)

2



1. Analyze live cell samples and follow the sorting strategies recommended by Markodimitraki et al., 2020 for live cells stained for DNA content.
2. Sort one cell per well in the 384-w CELseq2-containing primer plates from *step 1*.
3. After sorting of each plate is complete, seal it with proper aluminum seals (e.g. Greiner, 676090) and spin at 2000g for 1-2 minutes at 4°C . Freeze plates at -80°C .

scDam&T-seq molecular processing with robotic liquid handling

- 3 In the following steps, liquid dispersions of reaction mixes are performed using the Nanodrop II liquid handler (Innovadyne) and adapter dispersions are performed using the Mosquito LV (STP Labtech), as in the original protocol by Markodimitraki et al., 2020. It is recommended to use liquid handlers of similar range of dispersion volumes for the respective steps.

Note that many handlers require liquid "dead volume", which should be taken into account when preparing the master mix of each reaction. An example calculation sheet for the following scDam&T-seq liquid dispersions with the Nanodrop II is provided here:



DamT_Dispersions_Robots.xlsx 21.6KB

4 Lysis



This mix has to be dispensed as fast as possible after thawing the plates from -80°C .

Dispense 50 nL per well of Lysis mix:

Reagent	Volume (nL) per well
ultra-pure water	22.5
Spike-ins 1:50000	10
1% Igepal	7.5
dNTP mix 10mM each	10
TOTAL dispensation	50
Cumulative volume	100

Incubate at 65°C for 5 minutes in a PCR machine.

Place plates on ice and proceed with Reverse Transcription.

5 Reverse Transcription

Dispense 75 nL per well of RT mix:

Reagent	Volume (nL) per well
5X FS buffer	35
DTT 0.1M	17.5
RNase OUT (40 U/uL)	8.75
Superscript II (200 U/uL)	8.75
ultra-pure water	5
TOTAL dispensation	75
Cumulative volume	175

Incubate with the following program in a PCR machine:

42°C for 1h

4°C for 5 minutes

70°C for 10 minutes

Hold at 4°C

6 Second-strand synthesis

Dispense 925 nL per well of SSS mix:

Reagent	Volume (nL) per well
ultra-pure water	640
5X SS buffer	220
dNTP mix 10mM each	25
E. coli Ligase (10 U/uL)	7.5
DNA polymerase I (10 U/uL)	25
RNase H (2 U/uL)	7.5
TOTAL dispensation	925
Cumulative volume	1100

Incubate with the following program in a PCR machine:

16°C for 2 hours

Hold at 4°C

7 Proteinase K treatment



Dispense 250 nL per well of ProtK mix:

Reagent	Volume (nL) per well
ultra-pure water	50
10X Cutsmart buffer	135
Proteinase K solution (20 mg/mL)	65
TOTAL dispensation	250
Cumulative volume	1350

Incubate with the following program in a PCR machine:

50°C for 10h

80°C for 20 minutes

Hold at 4°C

8 DpnI digestion

Dispense 150 nL per well of DpnI mix:

Reagent	Volume (nL) per well
ultra-pure water	120
10X Cutsmart buffer	15
DpnI (20 U/uL)	15
TOTAL dispensation	150
Cumulative volume	1500

Incubate with the following program in a PCR machine:

37°C for 6 hours

80°C for 20 minutes

Hold at 4°C

9 Adapter ligation



Dispense **100 nL** per well DamID2 adapters from a 0.5uM motherplate, to a final adapter concentration of 25nM.

NOTE: This step is done with the Mosquito LV (STP Labtech) or equivalent liquid handler.

Dispense 400 nL per well of Ligation mix:

Reagent	Volume (nL) per well
ultra-pure water	150
Ligase buffer 10X	200
T4 Ligase	50
TOTAL dispensation	400
TOTAL cumulative volume	2000

Incubate with the following program in a PCR machine:

16°C for 16 hours

65°C for 10 minutes

Hold at 4°C

NOTE:

- The final DamID2 adapter concentration can be titrated lower, to improve the ratio between DamID and mRNA reads, especially for samples with low transcriptome.
- At this point plates can be frozen at -20°C until further processing.

Pooling, Amplification and Library preparation

10 All following steps of pooling, amplification by IVT and Illumina library preparation remain the same as what has been described by Markodimitraki et al., 2020.

11 Pooling

1. Pool the content of all wells of a 384-well plate by spinning it inverted on top of a collection reservoir at 500g for 2 minutes.
2. Using a p1000, carefully pipette the content from the collection plate to a 1.5mL or 2mL tube, trying to get as much as possible of the aqueous phase, which contains the barcoded DNA fragments. The oil phase can be pipetted out and thrown away after spinning the tube at max speed for 20-30 seconds. This should be repeated a few times until all the content of the plate is collected.
3. After all the content is collected, spin for 5-10 minutes at high speed.
4. Pipette out and discard any remaining oil phase from the top.
5. Pipette the aqueous phase and transfer to a clean tube, ensuring that you leave behind as much oil and debris as possible (debris usually remains as loose white precipitate at the bottom).
6. Spin the pooled material at high speed.
7. Repeat the transfer to a clean tube two more times.
8. During the last pipetting step measure the volume of the pooled material for each sample (henceforth one sample ~ material from one pooled plate).

NOTES:

- The final recovered volume (aqueous phase) should be at least 85% of the expected volume from a 384-w plate ($2\text{ uL/well} \times 384\text{ wells} = 768\text{ uL}$). Recovery of lower volumes does not necessarily indicate a failed experiment, but rather that dispensation errors may have occurred during processing, which may have affected the overall efficiency.
- To minimize loss of material when handling non-amplified samples, we recommend using **low-retention pipette tips**.

12 DNA purification

1. Per sample, add 0.8 volume beads diluted 1:10 in bead-binding buffer (see recipe in Materials).
2. Incubate for 20-30 minutes at room temperature.
3. Put samples on magnetic stand and incubate until all beads are bound to the magnet (liquid should look clear, around 20 minutes).
4. Remove unbound liquid.
5. Wash three times with freshly-made 80% ethanol.
6. During the last wash with ethanol, use a stronger hand magnet to concentrate the beads as much as possible at one place in the tube (they tend to be dispensed over the length of the tube)
7. Remove ethanol and let the beads air-dry until they look matte.
8. Elute in 7 ul ultra-pure water (DNase/RNase-free) for 10 minutes.
9. Transfer the eluted samples to PCR strips for the IVT reaction that follows.

NOTES:

- It is important that the beads don't over-dry before elution, as this can result in irreversible binding of fragments.
- It is not necessary to separate the eluted material from the beads for the IVT reaction.

13 Linear amplification by *in vitro* transcription (IVT)

Per sample add 9 uL of IVT mix, according to manufacturer instructions (MEGAScript T7 transcription kit):

Reagent	Volume (uL)
A	1.5
U	1.5
G	1.5
C	1.5
T7 buffer	1.5
T7 enzyme	1.5

Total reaction volume is 16 uL

Incubate with the following program in a PCR machine:

37°C for 14 hours

Hold at 4°C

The amplified RNA (aRNA) can be stored long-term at -80°C.



**14 aRNA purification**

1. Measure the exact volume of each aRNA sample with a pipette (should be around 15 uL) and transfer to a 1.5 mL tube.
2. Add ultra-pure water up to 30 uL.
3. Add 0.8 volume of undiluted beads. Incubate for 10 minutes.
4. Put samples on a magnetic stand and incubate until all beads are bound to the magnet (liquid should look clear).
5. Remove unbound liquid.
6. Wash three times with freshly-made 80% ethanol.
7. Air-dry beads until they look matte.
8. Elute in 20 uL ultra-pure water. Leave for 10 minutes.
9. Put on magnetic stand until clear and transfer the eluate in a clean tube.

15 aRNA fragmentation

1. Add 0.2 volume fragmentation buffer.
2. Incubate in a pre-heated block at 94°C for 90-120 seconds.
3. Transfer on ice and stop fragmentation with 0.1 volume 0.5M EDTA.

16 (repeat)**aRNA purification**

1. Add 0.8 volume of undiluted beads. Incubate for 10 minutes.
2. Put samples on a magnetic stand and incubate until all beads are bound to the magnet (liquid should look clear).
3. Remove unbound liquid.
4. Wash three times with freshly-made 80% ethanol.
5. Air-dry beads until they look matte.
6. Elute in 13 uL ultra-pure water. Leave for 10 minutes.
7. Put on magnetic stand until clear and transfer the eluate in a clean tube.

aRNA samples can be stored long-term at -80°C.

17 aRNA quantification

1. Measure 1 uL of aRNA on the Nanodrop to estimate total amount of product.
2. Run 1 uL of aRNA on the Bioanalyzer (Total Eukaryote RNA Assay) or equivalent.

NOTES:





- Based on the size distribution of the product, determine the ratio between aRNA and non-ligated adapter. If the adapter peak is more than twice as big as the aRNA product (see example below) it is necessary to perform extra bead clean-ups, to remove as much as possible of the non-ligated adapters. This is important as high amount of adapters may be amplified during library preparation and negatively affect the sequencing.
- Keep in mind that extra bead clean-up may entail some loss of product, so the decision whether and how many extra to perform should also depend on the amount of actual aRNA product you see on the Bioanalyzer at this first quantification.
- In case more bead clean-ups are needed, repeat *step 16* and make sure to do a quantification of the final aRNA product, before proceeding with library preparation.

18 Reverse transcription

1. Take 100ng of aRNA product diluted in 5 uL ultra-pure water. This amount can definitely be lower, in case the aRNA product is not abundant.
2. Add a mix of 1 uL Random Hexamer primer (20 uM) + 0.5 uL dNTPs (10mM).
3. Incubate @65°C for exactly 5 minutes.
4. Quickly transfer samples on ice.
5. Add 4 uL of RT mix:

Reagent	Volume (uL)
5X First-Strand buffer	2
DTT 0.1M	1
RNase OUT	0.5
Superscript II	0.5

Total reaction volume is 10.5 uL

Incubate with the following program in a PCR machine:

25°C for 10 minutes

42°C for 1 hour

Hold at 4°C

NOTE:

- The Random Hexamer primer sequence is GCCTTGGCACCCGAGAATTCCANNNNNN (Markodimitraki et al., 2020) and it includes the Illumina P7. Check guidelines on [illumina.com](https://www.illumina.com) for design.

19 Indexing PCR (based on Illumina Truseq small RNA)



1. Add 2 uL of a unique RPi primer (10uM) in each library.
2. Add 37.5 uL of PCR mix:

Reagent	Volume (uL)
2X NEBNext High Fidelity mastermix	25
RP1 primer 10uM	2
ultra-pure water	10.5

Total reaction volume is 50 uL

Incubate with the following program in a PCR machine:

98°C for 30 seconds

8-11 cycles of:

- 98°C for 10 seconds
- 60°C for 30 seconds
- 72°C for 30 seconds

72°C for 10 minutes

Hold at 4°C

NOTES:

- Each of the RPi primers (index primers) contains a unique index from the Illumina Truseq small RNA series (RPI series) and an overlapping sequence to the Illumina P7, introduced to the molecules during the previous RT step. Follow guidelines on illumina.com for design.
- The RP1 primer (universal primer) contains an overlapping sequence to the Illumina P5, which is part of the DamID2 adapters sequence. Follow guidelines on illumina.com for design.
- The exact number of cycles for the library PCR depends on the amount of input aRNA. We decide this empirically by comparing the height (FU) of the marker peak to the highest peak of the aRNA product distribution (excluding the adapter peak). For example, if the marker peak and the product are in similar FU levels, or if the product is much higher, we recommend 8 PCR cycles. Increase cycles accordingly for lower amounts.

20 Library purification



1. Add 0.8 volume of undiluted beads. Incubate for 10 minutes.
2. Put samples on a magnetic stand and incubate until all beads are bound to the magnet (liquid should look clear).

3. Remove unbound liquid.
4. Wash two times with freshly-made 80% ethanol.
5. Air-dry beads until they look matte.
6. Elute in 25 uL water. Leave for 10 minutes.
7. Put on magnetic stand until clear and transfer eluate in a clean tube.

(REPEAT)

1. Add 0.8 volume of undiluted beads. Incubate for 10 minutes.
2. Put samples on a magnetic stand and incubate until all beads are bound to the magnet (liquid should look clear).
3. Remove unbound liquid.
4. Wash two times with freshly-made 80% ethanol.
5. Air-dry beads until they look matte.
6. Elute in 13 uL water. Leave for 10 minutes.
7. Put on magnetic stand until clear and transfer eluate in a clean tube.

Purified DNA libraries can be stored long-term at -20°C .

21 **Library quantification**

1. Measure 1-2 uL of library with Qubit dsDNA High Sensitivity assay to determine total library amount.
2. Run a max of 2ng of library in a Bioanalyzer (High Sensitivity DNA Assay) or equivalent to estimate size distribution.
3. Calculate library molarity based on Qubit concentration and size distribution.



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